HaloPlex Target Enrichment System

For Illumina Sequencing

Protocol

Version A, February 2012

Research Use Only. Not for use in Diagnostic Procedures.

Agilent Technologies

Notices

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In this Guide...

This guide describes an optimized protocol for using the Agilent HaloPlex target enrichment system to prepare sequencing library samples for Illumina paired-end multiplexed sequencing platforms.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation

This chapter describes the steps of the HaloPlex workflow, to prepare target-enriched sequencing libraries for the Illumina platform.

3 Reference

This chapter contains reference information, including component kit contents and index sequences.

Content

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE Agilent cannot guarantee the HaloPlex Target Enrichment Kits and cannot provide technical support for the use of non-Agilent protocols or reagents to process samples for enrichment.

Procedural Notes

- The HaloPlex target enrichment protocol is configured for processing samples in sets of 12 samples per run. A 96 reaction kit contains enough reagents to prepare master mixes for eight runs of 12 samples each. When processing samples using runs with fewer than 12 samples, some reagents may be depleted before 96 samples are run.
- The HaloPlex protocol is optimized for digestion of 800 ng of genomic DNA (split among 8 different restriction digestion reactions) plus 100 ng excess DNA, for a total of 900 ng genomic DNA. Using lower amounts of DNA in the enrichment protocol can adversely affect your results. Use a fluorometry-based DNA quantitation method, such as PicoGreen stain or Qubit fluorometry to quantify the DNA starting material.
- Always keep pre-amplification and post-amplification DNA samples in separate work areas. Perform the enrichment procedure in the pre-amplification area. Open and store the amplified, enriched DNA samples only in the post-amplification area.
- Possible stopping points, where DNA samples may be stored overnight, are marked in the protocol. Store the samples at –20°C, but do not subject the samples to multiple freeze/thaw cycles.
- Ensure that master mixes are thoroughly mixed, by pipetting up-and-down or by gentle vortexing, before distributing to the samples.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for HaloPlex Target Enrichment

Description	Vendor and part number
HaloPlex Target Enrichment System Kit	Agilent
16 reactions	p/n G9900A
96 reactions	p/n G9900B
480 reactions	p/n G9900C
Phusion Hot Start II High-Fidelity DNA Polymerase	Finnzymes p/n F-549L
dNTPs, 25 mM for each nucleotide	Agilent p/n 200415
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
10 mM Tris-HCl, pH 8.0 or 10 mM Tris-acetate, pH 8.0	General laboratory supplier
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Novex 6% Polyacrylamide, TBE Pre-cast Gels	Life Technologies p/n EC62655BOX
Novex TBE Running Buffer, 5X	Life Technologies p/n LC6675
Novex High-density TBE Sample Buffer, 5X	Life Technologies p/n LC6678
GelRed Nucleic Acid Stain, 3X in water	Biotium p/n 41001
DNA molecular weight markers	General laboratory supplier
Quant-iT dsDNA BR Assay Kit, for use with the Qubit fluorometer	
100 assays, 2-1000 ng	Life Technologies p/n Q32850
500 assays, 2-1000 ng	Life Technologies p/n Q32853

Required Equipment

Table 2 Required Equipment for HaloPlex Target Enrichment

Description	Vendor and part number
Thermal Cycler	Agilent SureCycler 8800, p/n G8800A 96 well plate module, p/n G8810A compression mats, p/n 410087 or equivalent thermal cycler and accessories
Thermal cycler-compatible 96-well plates	Agilent p/n 401333 (for SureCycler 8800) or see manufacturer's recommendations
8-well PCR strip tubes with caps	Nippon Genetics, p/n FG-088WF, or equivalent
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
XCell SureLock Mini-cell for gel electrophoresis	Life Technologies p/n EI0001
Benchtop microcentrifuge	VWR p/n 93000-196, or equivalent
Benchtop plate centrifuge	Labnet International MPS1000 Mini Plate Spinner p/n C1000, or equivalent
Qubit 2.0 Fluorometer	Life Technologies p/n Q32866
Qubit assay tubes	Life Technologies p/n Q32856
96-well plate-compatible magnetic separator	Agencourt SPRIPlate Super Magnet Plate p/n A32782
1.5 mL tube-compatible magnetic separator	DynaMag-2 magnet, Life Technologies p/n 12321D, or equivalent
Multichannel pipettes (10-µL and 100-µL volume)	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Adhesive seals for 96-well PCR plates	Agilent p/n 410186, or equivalent
Ice bucket	General laboratory supplier
Vortex mixer	General laboratory supplier

2 Sample Preparation

This section contains instructions for gDNA library target enrichment for sequence analysis using the Illumina platform. For each sample to be sequenced, an individual target-enriched, indexed library is prepared.

The target region can vary from 1 to 500 kb. The custom HaloPlex probe supplied with each kit must be designed before purchasing the kit using the online HaloPlex Design Wizard at www.agilent.com/genomics/ngs.

The HaloPlex Target Enrichment System amplifies thousands of targets in the same reaction, incorporating standard Illumina paired-end sequencing motifs in the process. During the amplification, each sample can be uniquely indexed, allowing for pooling of up to 96 samples per sequencing lane.

See Figure 1 for a summary of the overall HaloPlex target enrichment workflow.

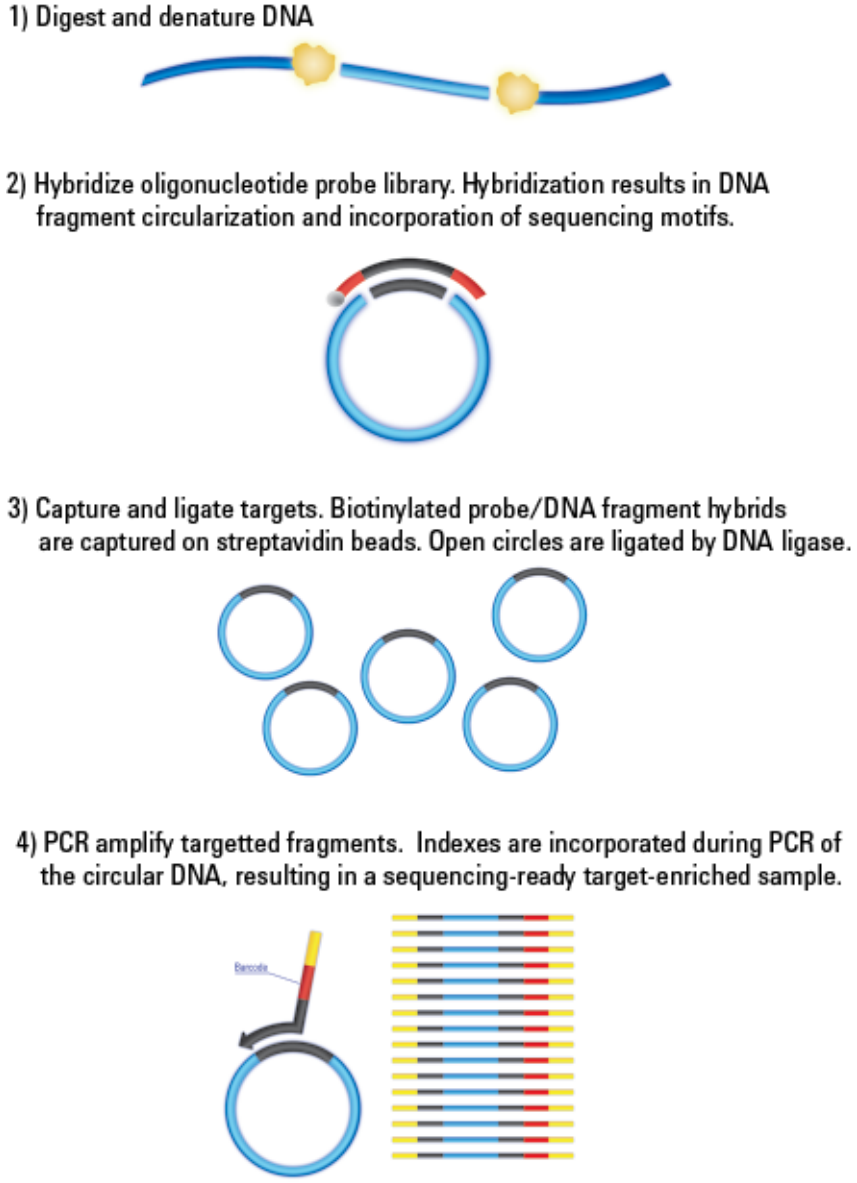


Figure 1 Overall HaloPlex target-enriched sequencing sample preparation workflow.

Step 1. Digest genomic DNA with restriction enzymes

In this step, the gDNA sample is aliquoted to eight digestion reactions, each containing two restriction enzymes. After digestion, the eight reactions are pooled, resulting in a single DNA sample containing a 16-enzyme restriction fragment library that includes both target and non-target gDNA regions.

NOTE

Successful enrichment requires high-quality and carefully quantified DNA samples.

Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0. Verify the size distribution of DNA in each DNA preparation by gel electrophoresis. Any smearing below 2.5 kb indicates sample degradation.

In the protocol below, 800 ng genomic DNA is split among eight different restriction digests, with an additional 100 ng excess DNA included to allow for pipetting losses. Using <900 ng DNA in the enrichment protocol can result in low yield and can potentiate rare allele dropouts. Use a fluorometry-based DNA quantitation method, such Qubit fluorometry or PicoGreen staining, to accurately quantify the DNA starting material.

1 Use the Qubit dsDNA BR Assay or PicoGreen staining kit to determine the concentration of your gDNA sample.

   Follow the manufacturers instructions for the kits and instruments.

2 Transfer 45 µL of the supplied Enrichment Control DNA to a 0.2-mL PCR tube. Store on ice.

3 In separate 0.2-mL PCR tubes, dilute 900 ng of each gDNA sample in 45 µL nuclease-free water, for a final DNA concentration of 20 ng/µL. Store sample tubes on ice.

Prepare the appropriate number of gDNA samples for a single run:

- 96-reaction kits and 480-reaction kits are configured for sample processing in sets of 12 samples (11 gDNA samples + 1 control DNA sample) per run.
- 16-reaction kits may be used for sample processing in sets of up to 16 samples (15 gDNA samples + 1 control DNA sample) per run.

NOTE

All instructions in the following section apply to 12-sample runs. When processing >12 samples, adjust the volumes of components added to master mixes accordingly, and set up an additional restriction digest reaction plate for samples 13 to 16.

Volumes specified in the following protocol are for 12 samples plus 1 reaction volume excess (13 reaction equivalents total). Calculate the amount of each solution needed for the number of reactions in your run, plus one reaction excess.

For example, preparation of the Restriction Enzyme Master Mix strip on page 16 requires 52 µL of RE buffer for 13 reaction equivalents, or 4 µL per reaction. For 16-reaction runs, use 17 reaction equivalents, or 68 µL of RE Buffer.

4 Prepare the Restriction Enzyme Master Mix strip.

In this step, eight separate restriction enzyme master mixes are prepared by combining two enzymes (from Enzyme Strips 1 and 2) and restriction buffer in each well of an 8-well strip tube. Figure 2 illustrates how to prepare the Restriction Enzyme Master Mix strip using the steps detailed on page 16.

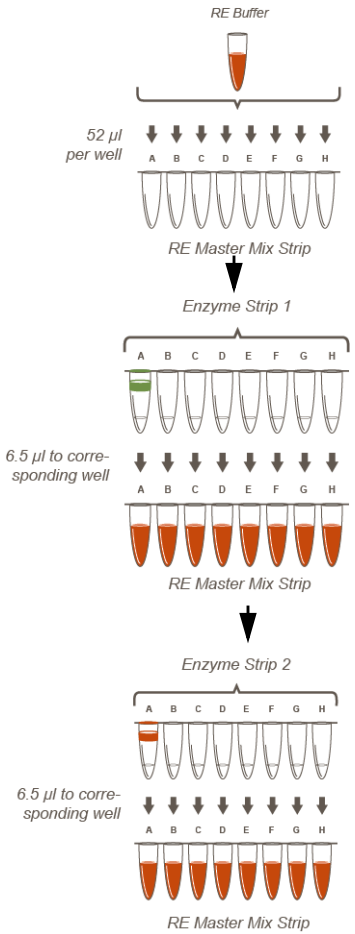


Figure 2 Preparation of the Restriction Enzyme Master Mix Strip.

a Add 52 µL of RE Buffer to each tube of an 8-well strip tube.

b Using a multichannel pipette, add 6.5 µL of each enzyme from tubes A to H of Enzyme Strip 1 (with green marker on tube A) to each corresponding tube A to H of the master mix strip tube.

c Using a multichannel pipette, add 6.5 µL of each enzyme from tubes A to H of Enzyme Strip 2 (with red marker on tube A) to each corresponding tube A to H of the master mix strip tube.

d Mix by gentle vortexing and then spin briefly to collect the liquid.

e Keep the Restriction Enzyme Master Mix Strip on ice until it is used in step 5.

5 Aliquot the Restriction Enzyme Master Mixes to a 96-well restriction digest reaction plate.

a Align the Restriction Enzyme Master Mix Strip, prepared in step 4, along the vertical side of a 96-well PCR plate as shown below.

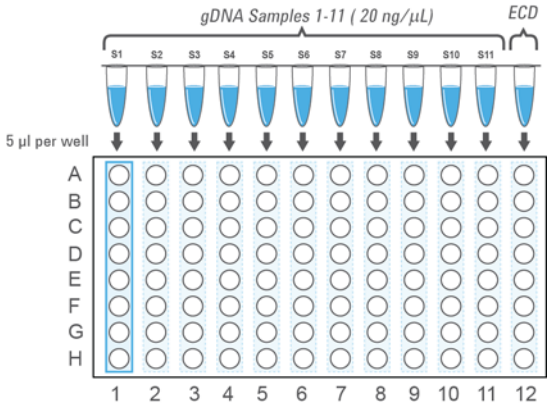
b Using a multichannel pipette, carefully distribute 5 µL of each RE master mix row-wise into each well of the digestion reaction plate.

Visually inspect pipette tips for equal volumes before dispensing to the plate.

Each row of the 96-well plate now contains 5 µL per well of the same restriction enzyme combination.

6 Aliquot gDNA samples into the 96-well restriction digest reaction plate.

a Align the 12 DNA samples (11 gDNA samples and the ECD sample), prepared in step 2 and step 3, along the horizontal side of the digestion reaction plate as shown below.



b Using a multichannel pipette, carefully distribute 5 µL of DNA samples column-wise into each well of the digestion reaction plate.

Visually inspect pipette tips for equal volumes before dispensing.

c Seal the plate thoroughly with adhesive plastic film.

The prepared 96-well plate now contains complete 10-µL restriction digestion reactions. In this format, each column of the plate corresponds to one DNA sample digested in eight different restriction reactions.

7 Briefly spin the sealed plate in a plate centrifuge.

8 Carefully vortex the plate to mix the digestion reactions.

9 Briefly spin the plate in a plate centrifuge.

10 Place the plate in a thermal cycler and run the program in Table 3, using a heated lid.

Table 3 Thermal cycler program for HaloPlex restriction digestion

Step	Temperature	Time	Purpose
Step 1	37°C	4 hours	DNA digestion
Step 2	80°C	20 minutes	Enzyme inactivation
Step 3	4°C	Hold	Hold

11 Validate the restriction digestion reaction by gel analysis of the Enrichment Control DNA reaction. The Enrichment Control DNA sample contains genomic DNA mixed with an 800-bp PCR product that contains restriction sites for all the enzymes used in the digestion protocol.

- **a** Using a multichannel pipette, carefully transfer 4 µL of each Enrichment Control DNA digestion reaction from wells A12 to H12 of the reaction plate to fresh 0.2-mL PCR tubes.
- **b** Prepare an undigested DNA gel control by combining 2 µL of the Enrichment Control DNA stock solution and 2 µL of nuclease-free water in a separate tube.
- **c** Add 1 µL of Novex Hi-Density TBE Sample Buffer (5X) to each sample.
- **d** Prepare the XCell SureLock Mini-Cell with a 12-well Novex 6% polyacrylamide TBE pre-cast gel. Fill with 1X Novex TBE Running Buffer.
- **e** Load 5 µL of each sample on the gel. In one or more adjacent lanes, load 200 ng of a 50-bp DNA ladder.
- **f** Run the gel at 210 V for 15 minutes.
- **g** Stain the gel in 3X GelRed Nucleic Acid Stain for 10 minutes, and visualize bands under UV radiation.

The undigested control should have gDNA bands >2.5 kbp and a PCR product band at 800 bp. Digested samples should have a smear of gDNA restriction fragments between 100 and 2500 bp, overlaid with three distinct bands at 125, 225, and 450 bp. See Figure 3 for a sample gel.

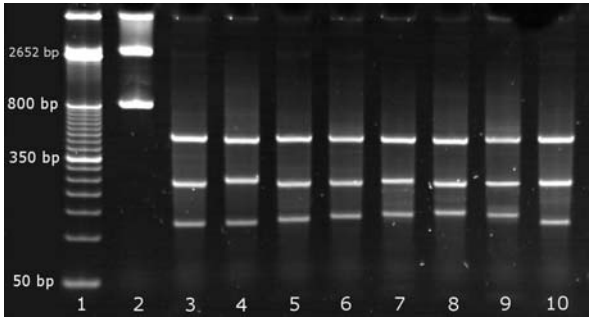


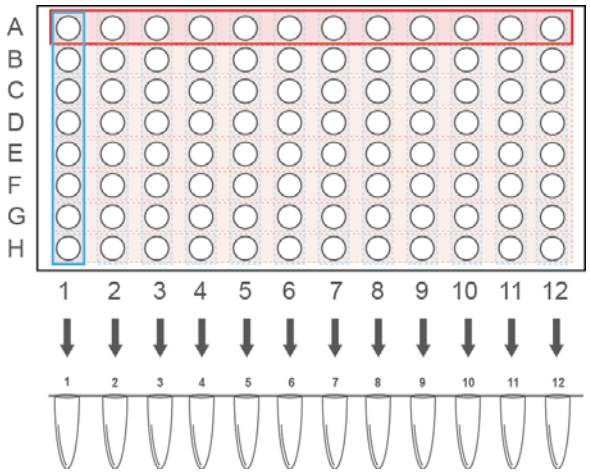
Figure 3 Validation of restriction digestion. Lane 1: 50-bp DNA ladder, Lane 2: Undigested Enrichment Control DNA, Lanes 3–10: ECD digestion reactions 1–8.

12 Pool the eight 10-µL restriction digest reactions for each gDNA sample, column-wise, into a 0.2-mL PCR tube as shown in the figure below.

NOTE

Due to partial evaporation of samples, you may recover less than 10 µL of each restriction digest. Minor reductions to the digested DNA pool volume will not impact hybridization performance; you do not need to compensate for any sample evaporation volume losses in the final pool.

13 Pool the remaining 6 µL from each Enrichment Control DNA digest into a separate tube as shown in the figure below. Add 32 µL of nuclease-free water to compensate for the volume removed for gel analysis.



Stopping Point If you do not continue to the next step, samples may be stored at –20°C for long term storage. There are no more stopping points until after the PCR amplification step on page 28.

Step 2. Hybridize digested DNA to HaloPlex probes

In this step, the collection of gDNA restriction fragments is hybridized to the HaloPlex probe capture library. HaloPlex probes are designed to hybridize selectively to fragments originating from target regions of the genome and to direct circularization of the targeted DNA fragments. During the hybridization process, Illumina sequencing motifs are incorporated into the targeted fragments.

1 Prepare a Hybridization master mix by combining the reagents in the following table.

   Mix well by gentle vortexing, then spin the tube briefly.

Table 4 Hybridization Master Mix

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
Hybridization Solution	65 µL	845 µL
Primer Cassette	1 µL	13 µL
HaloPlex Probes	14 µL	182 µL
Total Volume	**80 µL**	**1040 µL**

2 Add 80 µL of the Hybridization Master Mix to each digested DNA sample.

3 Vortex the reactions briefly and then spin tubes briefly.

4 Place the tubes in a thermal cycler and run the program in Table 5, using a heated lid.

Table 5 Thermal cycler program* for HaloPlex probe hybridization

Step	Temperature	Time
Step 1	95°C	10 minutes
Step 2	75°C	30 minutes
Step 3	68°C	30 minutes
Step 4	62°C	30 minutes
Step 5	55°C	30 minutes
Step 6	46°C	10 hours
Step 7	8°C	Hold†

* Thermal cyclers that use calculated temperature methods cannot be set to 160 µL reaction volumes. In that case, enter the maximum possible volume.

† Samples can be held at 8°C or 4°C for up to 72 hours prior to capture.

Step 3. Capture the target DNA

In this step, the circularized target DNA-HaloPlex probe hybrids, containing biotin, are captured on streptavidin beads.

1 Remove reagents to be used in upcoming protocol steps from cold storage and allow the solutions to reach room temperature:

- From –20°C storage, remove the Capture Solution, Wash Solution, Ligation Solution, Haloase A Buffer, and Haloase B buffer.
- From +4°C storage, remove the Magnetic Beads.

2 Vigorously resuspend the provided Magnetic Beads on a vortex mixer. The magnetic beads settle during storage.

3 Prepare 20 µL (1 Volume) of Magnetic Beads per hybridization sample, plus excess, for the capture reaction:

a For 12-sample runs, transfer 250 µL of the bead suspension to a 1.5-mL tube.

For runs that include greater or fewer than 12 samples, adjust the volumes of the bead suspension and Capture Solution accordingly.

b Put the tube into a 1.5 mL tube-compatible magnetic rack for 5 minutes.

c Wait for the solution to clear, then carefully remove and discard the supernatant using a pipette.

d Add 2 Volumes (500 µL for 12-sample runs) of Capture Solution to the beads and resuspend by pipetting up and down.

4 Add 40 µL of the prepared bead suspension to each 160-µL hybridization reaction.

NOTE

When adding beads to the hybridization reactions, visually inspect the bead preparation to ensure a homogenous suspension with no aggregated bead mass at the bottom of the tube. If aggregation is present, thoroughly resuspend the beads by vortexing and pipetting up and down before use.

5 After adding the magnetic beads, mix the capture reactions thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 80 µL.

6 Incubate the capture reactions at room temperature for 15 minutes.

7 Briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the Agencourt SPRIPlate Super Magnet magnetic rack.

NOTE Use the Agencourt SPRIPlate Super Magnet magnetic rack for the remainder of magnetic bead collection steps for samples in tube strips or PCR tubes.

8 Wait for the solution to clear (about 30 seconds), then remove and discard the supernatant using a pipette set to 200 µL.

9 Wash the bead-bound samples:

a Remove the capture reaction tubes from the magnetic rack and add 100 µL of Wash Solution to each tube.

b Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 80 µL.

c Incubate the tubes in a thermal cycler at 46°C for 10 minutes, using a heated lid.

The thermal cycler may be programmed to include a 4°C hold step following the 10-minute incubation.

d Briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the magnetic rack.

e Wait for the solution to clear (about 30 seconds), then carefully remove and discard the supernatant using a pipette set to 120 µL. If necessary, carefully remove any residual liquid with a 20-µL volume pipette.

Step 4. Ligate the captured, circularized fragments

In this step, DNA ligase is added to the capture reaction to catalyze the formation of closed-circular DNA from the circularized target DNA-HaloPlex probe hybrids. Non-target DNA in the capture reaction liquid phase remains in linear fragment form.

1 Prepare a DNA ligation master mix by combining the reagents in the following table. Mix the components thoroughly by pipetting up and down.

Table 6 Preparation of DNA ligation master mix

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
Ligation Solution	47.5 µL	617.5 µL
DNA Ligase	2.5 µL	32.5 µL
Total Volume	**50 µL**	**650 µL**

2 Add 50 µL of the DNA ligation master mix to the beads in each DNA capture reaction tube.

3 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 40 µL.

4 Incubate the tubes in a thermal cycler at 55°C for 10 minutes, using a heated lid.

The thermal cycler may be programmed to include a 4°C hold step following the 10-minute incubation.

5 Briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the magnetic rack.

6 Wait for the solution to clear (about 30 seconds), then carefully remove and discard the supernatant using a pipette set to 70 µL. If necessary, carefully remove any residual liquid with a 20-µL volume pipette.

Step 5. Remove non-target DNA by Haloase A and B digestion

In this step linear, non-target DNA fragments are digested by the exonuclease activity of the provided Haloase A and B enzymes. During the Haloase B treatment, circularized target DNA is released from the streptavidin beads into the liquid phase.

1 Prepare the Haloase A master mix by combining the reagents in the following table. Mix the components thoroughly by pipetting up and down.

Table 7 Preparation of Haloase A master mix

Reagent	Volume for 1 Reaction	Volume for 12 Reactions (includes excess)
Haloase A Buffer	22.5 µL	315 µL
Haloase A1	0.5 µL	7 µL
Haloase A2	2.0 µL	28 µL
Total Volume	**25 µL**	**350 µL**

2 Add 25 µL of the Haloase A master mix to the beads in each DNA capture reaction tube.

3 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 15 µL.

4 Incubate the tubes in a thermal cycler at 37°C for 30 minutes, using a heated lid.

The thermal cycler may be programmed to include a 4°C hold step following the 30-minute incubation.

5 Briefly spin the tubes in a desktop centrifuge and then transfer the tubes to the magnetic rack.

6 Wait for the solution to clear (about 30 seconds), then carefully remove and discard the supernatant using a 100-µL pipette set to 50 µL. If necessary, carefully remove any residual liquid with a 20-µL volume pipette.

7 Add 21.5 µL of Haloase B Buffer to the beads in each DNA capture reaction tube.

8 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 15 µL.

9 Incubate the tubes in a thermal cycler at 80°C for 20 minutes, using a heated lid, to inactivate any residual Haloase A activity.

10 Remove the tubes from the thermal cycler, and wait for the reactions to cool to room temperature.

11 Add 3.5 µL of Haloase B to the 21.5-µL bead suspension in each reaction tube.

12 Resuspend the beads thoroughly by pipetting up and down 10 times using a 100-µL multichannel pipette set to 15 µL.

13 Incubate the tubes in a thermal cycler at 37°C for 30 minutes, using a heated lid.

The thermal cycler may be programmed to include a 4°C hold step following the 30-minute incubation.

During the 30-minute incubation, prepare the PCR master mix used in the following step.

Step 6. Amplify and index captured target libraries

In this step, the captured DNA is amplified and index-tagged in PCR reactions containing Illumina Primer 1.0 along with the appropriate Index Primer.

For sample indexing primer assignments, see "Nucleotide Sequences of HaloPlex Indexes" on page 38 for nucleotide sequences of the 96 indexes used in the HaloPlex Target Enrichment System. For low-plexity pooling, assign indexes row-wise from the plate starting with Index A1.

NOTE

Steps 1-3 below should be completed during the 30-minute Haloase B reaction period described on page 26. When the Haloase B incubation is complete, transfer the supernatant directly into the prepared PCR reaction tubes.

1 Prepare the PCR master mix by combining the reagents in the following table.

Table 8 Preparation of PCR master mix

Reagent	Stock Concentration	Volume for 1 reaction	Volume for 12 reactions (includes excess)
Phusion HF Buffer	5×	6 µL	84 µL
dNTPs	25 mM	0.4 µL	5.6 µL
Primer 1.0	25 µM	1 µL	14 µL
Phusion HF DNA Polymerase	2 U/µL	0.5 µL	7 µL
Nuclease-free water		12.1 µL	169.4 µL
Total		**20 µL**	**280 µL**

2 Mix the master mix components by gentle vortexing, then distribute 20-µL aliquots to fresh 0.2-mL reaction tubes.

3 Add 10 µL of the appropriate Index Primer to each tube. Be sure to record the specific Index Primer added to each tube for later sequence analysis.

4 When the 30-minute Haloase B reaction period is complete, briefly spin the Haloase B reaction tubes in a desktop centrifuge and then transfer the tubes to the magnetic rack.

5 Wait for the solution to clear (about 30 seconds), then carefully transfer 20 µL of the supernatant to the 30-µL PCR reaction tube that contains the appropriate Index Primer. Mix by pipetting up and down or by gentle vortexing.

6 Run the program in Table 9 in a thermal cycler, using a heated lid.

The optimal amplification cycle number varies for each custom HaloPlex Probe design. Consult the Certificate of Analysis (provided with HaloPlex Target Enrichment System Box 1) for the PCR cycling recommendation for your custom probe.

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Table 9

Segment	Number of Cycles	Temperature	Time
1	1	98°C	30 seconds
2	Obtain cycle number from Certificate of Analysis	98°C	10 seconds
		65°C	30 seconds
		72°C	30 seconds
3	1	72°C	5 minutes
4	1	8°C	Hold

Stopping Point If you do not continue to the next step, PCR products may be stored at –20°C for up to 72 hours, For best results, however, purify PCR products as soon as possible.

Step 7. Purify the amplified target libraries

In this step, the amplified target DNA is purified using AMPure XP beads.

1. Let the AMPure XP beads come to room temperature for at least 30 minutes.
2. Transfer 40 µL of each PCR reaction sample to a fresh tube. Store the remaining volume of each sample at –20°C for troubleshooting.
3. Mix the bead suspension well, until the suspension appears homogeneous and consistent in color.
4. Add 60 µL (1.5 Volumes) of homogenous AMPure XP beads to each 40-µL amplified library sample. Pipette up and down to mix.

   Using a bead to sample volume ratio of 1.5:1 is imperative to ensure optimal purification results.
5. Mix the reactions thoroughly by pipetting up and down 15 times using a 100-µL pipette set to 80 µL.
6. Incubate samples for 5 minutes at room temperature.
7. Put the tube into a magnetic rack. Wait for the solution to clear (approximately 2 minutes).
8. Keep the tube in the magnetic rack. Carefully remove and discard the cleared solution from each well using a 100-µL pipette set to 100 µL. Do not touch the beads while removing the solution.
9. Continue to keep the tube in the magnetic rack while you add 100 µL of 70% ethanol into the tube

   Use fresh 70% ethanol for optimal results.
10. Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol using a 100-µL pipette set to 100 µL.
11. Repeat step 9 and step 10 step once for a total of two washes.
12. Remove any residual ethanol with a 20-µL volume pipette.
13. Air-dry the tubes with open lids at room temperature until the residual ethanol completely evaporates.

    Make sure all ethanol has evaporated before continuing.
14. Remove tubes from the magnetic rack and add 40 µL of 10 mM Tris-acetate or Tris-HCl buffer (pH 8.0) to each sample.
15. Mix thoroughly by pipetting up and down 15 times using a 100-µL pipette set to 30 µL.

16 Incubate for 2 minutes at room temperature to allow elution of DNA.

17 Put the tube in the magnetic rack and leave for 2 minutes or until the solution is clear.

18 Remove the cleared supernatant (approximately 40 µL) to a fresh tube. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, samples may be stored at –20°C for long-term storage (up to one year). Avoid subjecting the stored DNA samples to multiple freeze-thaw cycles.

The HaloPlex target-enriched sequencing library samples are now ready for validation and pooling for multiplex sequencing. See page 31 to page 33 for recommended validation procedures and for pooling guidelines.

Each amplicon in the prepared library contains one target insert surrounded by sequence motifs required for multiplexed sequencing using the Illumina platform. The amplicons should range from 225 to 525 bp in length, including 100 to 400 bp of target DNA insert and 125 bp of sequencing motifs.

Figure 4 Content of HaloPlex-enriched target amplicons. Each amplicon contains one target insert (blue) surrounded by the Illumina paired-end sequencing elements (black), the sample index (red) and the library bridge PCR primers (yellow).

Step 8. Optional: Validate enrichment by gel analysis

Prior to sample pooling and sequencing sample preparation, verify successful enrichment by gel analysis of the Enrichment Control DNA sample and a selection of your library samples.

1. Transfer 5 µL of the captured library prepared from the Enrichment Control DNA to a fresh 0.2-mL PCR tube.
2. Transfer 5 µL of a selection of enriched libraries prepared from your samples to separate fresh 0.2-mL PCR tubes.
3. Add 2 µL of Novex Hi-Density TBE Sample Buffer (5X) to each sample.
4. Prepare the XCell SureLock Mini-Cell with a 12-well Novex 6% polyacrylamide TBE pre-cast gel. Fill with 1X Novex TBE Running Buffer.
5. Load 7 µL of each sample on the gel. In one or more adjacent lanes, load 200 ng of a DNA ladder.
6. Run the gel at 210 V for 15 minutes.
7. Stain the gel in 3X GelRed Nucleic Acid Stain for 10 minutes, and visualize bands under UV radiation.

Successful enrichment is indicated by the presence of a smear of amplicons from approximately 225 to 525 bp in each enrichment library lane. See Figure 3 for a sample lane image.

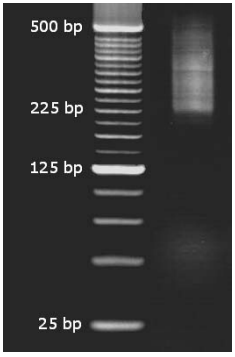

Figure 5 Validation of HaloPlex enrichment process. Lane 1: 25-bp DNA ladder, Lane 2: Enrichment Control DNA enriched library sample.

Step 9. Optional: Validate and quantify target libraries using the 2100 Bioanalyzer

Use a Bioanalyzer High Sensitivity DNA Assay kit and the Agilent 2100 Bioanalyzer with Agilent 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit). See the reagent kit guide for general Bioanalyzer instrument and assay setup instructions.

1. Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of captured library samples for the analysis.
2. Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
3. Check that the electropherogram shows a fragment size distribution between approximately 225 to 525 bp. Sample electropherograms for libraries prepared using three different probe designs are shown in Figure 6.
4. Determine the concentration of each library pool by integration under the peak in the electropherogram.

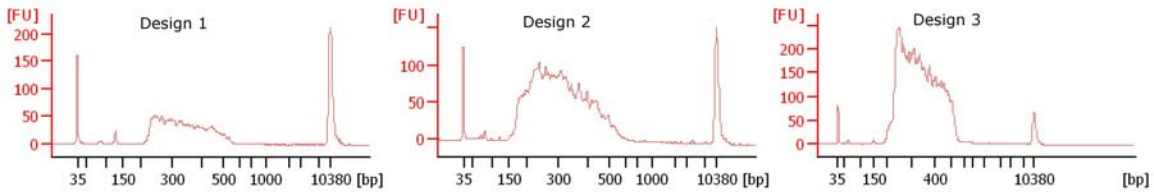

Figure 6 Analysis of amplified enrichment products from three custom probe designs using the Agilent Bioanalyzer High Sensitivity DNA Assay. The electropherograms show a DNA size distribution of approximately 225 to 525 bp. Some iterated designs have non-default size distributions, such as the sample enriched using Design 2, which contains amplicons from 175 to 525 bp.

Step 10. Pool samples with different indexes for multiplexed sequencing

Use the following guidelines to design your sample pooling strategy:

- Determine the DNA concentration in each enriched sample. The expected output concentration is at least 10 nM.
- Pool equimolar amounts of each sample to optimize the use of sequencing capacity.
- The average amplicon length in any design is approximately 335 bp.
- The final HaloPlex enrichment pool is ready for direct sequencing using standard Illumina paired-end primers and chemistry on the Illumina HiSeq, MiSeq, or GAIIx platform, depending on the platform selected during probe design.

3 Reference

This chapter contains reference information, including component kit contents and index sequences.

Kit Contents

The HaloPlex Target Enrichment System includes the following component kits:

Table 10 HaloPlex Target Enrichment System Kit Contents

Component Kits	Storage Condition	16 Reactions	96 Reactions	480 Reactions
HaloPlex Target Enrichment System 1-500 kb, ILM Box 1*	–20°C†	5190-5017	5190-5021	5 × 5190-5311
HaloPlex Target Enrichment System 1-500 kb, ILM Box 2‡	+4°C	5190-5015	5190-5019	5 × 5190-5019

* See Table 11 for list of included reagents.

† For long-term storage (>30 days), store Enzyme Strip 1 and Enzyme Strip 2 at –70°C.

‡ Contains Magnetic Beads.

The contents of the HaloPlex Target Enrichement System Box #1 are detailed in the table below.

Table 11 HaloPlex Target Enrichment System1-500 kb, ILM Box 1 Contents

Included Reagents	16 Reaction Kit	96 Reaction Kit	480 Reaction Kit
Hybridization Solution	tube with clear cap	bottle	bottle
Ligation Solution	tube with clear cap	bottle	bottle
Wash Solution	tube with clear cap	bottle	bottle
Capture Solution	tube with clear cap	bottle	bottle
DNA Ligase	tube with red cap	tube with red cap	tube with red cap
RE Buffer	tube with clear cap	bottle	bottle
Haloase A1	tube with green cap	tube with green cap	tube with green cap
Haloase A2	tube with blue cap	tube with blue cap	tube with blue cap
Haloase A Buffer	tube with clear cap	bottle	bottle
Haloase B	tube with black cap	tube with black cap	tube with black cap
Haloase B Buffer	tube with clear cap	bottle	bottle
Enrichment Control DNA	tube with orange cap	tube with orange cap	tube with orange cap
Primer Cassette	tube with purple cap	tube with purple cap	tube with purple cap
Primer 1.0	tube with yellow cap	tube with yellow cap	tube with yellow cap
Index Primers	16 tubes containing Index Primers A1-H2	96-well plate with Index Primers A1-H12	5 × 96-well plates with Index Primers A1-H12
Enzyme Strip 1	8-well strip tube with green label	8-well strip tube with green label	8-well strip tube with green label
Enzyme Strip 2	8-well strip tube with red label	8-well strip tube with red label	8-well strip tube with red label
HaloPlex Probe	tube with pink cap	tube with pink cap	tube with pink cap

Nucleotide Sequences of HaloPlex Indexes

The nucleotide sequence of the index portion of each HaloPlex Index Primer is provided in the tables below. HaloPlex 16-reaction kits include tubes containing the 16 primers listed in Table 12. The 96- and 480-reaction kits include plates containing the 96 indexes listed (in column-wise order) in Table 12 to Table 17.

Table 12 HaloPlex Indexes A1-H2

Index Number	Sequence
A1	CTCGGT
B1	TTACGG
C1	GCGTCC
D1	GAGTAT
E1	ATATAC
F1	GGCCTT
G1	CCGTTC
H1	ATGGTA
A2	AATCGT
B2	CCTTCG
C2	AGGTAC
D2	AGCTAT
E2	GCCGAC
F2	AGACGT
G2	TACTTC
H2	GTACGA

Table 13 HaloPlex Indexes A3-H4

Index Number	Sequence
A3	GCGCGT
B3	GGAGCG
C3	ACGTTA
D3	CAAGAT
E3	CTTAAC
F3	CATAGT
G3	GAGGTC
H3	AAGAGA
A4	CGAAGT
B4	ACGCAG
C4	AACCTA
D4	TCGTTG
E4	GTTCTA
F4	GATGAT
G4	ATCCTC
H4	GGCAGA

Table 14 HaloPlex Indexes A5-H6

Index Number	Sequence
A5	TATTCT
B5	TGCCAG
C5	TGGATA
D5	ACTCTG
E5	CAGCTA
F5	CCTATG
G5	TCAATC
H5	GGAGAA
A6	AGATCT
B6	GAGAAG
C6	TTATCA
D6	GATATG
E6	ACCGGA
F6	AACTGG
G6	CTTCGC
H6	GCGCAA

Table 15 HaloPlex Indexes A7-H8

Index Number	Sequence
A7	CAGGCT
B7	ATCAAG
C7	ATAGAA
D7	TATGCG
E7	CTCCGA
F7	GCGAGG
G7	GACCGC
H7	GCGGTT
A8	TCCGCT
B8	CGATTC
C8	CTGGTT
D8	GTACCG
E8	TTAAGA
F8	TTCTCG
G8	ATAAGC
H8	TTAGTT

Table 16 HaloPlex Indexes A9-H10

Index Number	Sequence
A9	GGTCCT
B9	ACCGTC
C9	GGAGTT
D9	CAGACG
E9	GGTTCA
F9	GCTGCG
G9	CATTAC
H9	AGAATT
A10	TCGTAT
B10	TAAGTC
C10	TACCTT
D10	CCTGAG
E10	ACGCCA
F10	CTGGCG
G10	TGATAC
H10	ATCAGT

Table 17 HaloPlex Indexes A11-H12

Index Number	Sequence
A11	GTCCAT
B11	TTCATC
C11	TCTACT
D11	TATTGC
E11	CGACCA
F11	CGAACG
G11	CTAGAC
H11	GGCGCT
A12	GATTGG
B12	AGCAGC
C12	ATAACT
D12	GAGAGC
E12	TCGGAA
F12	ATTCAG
G12	TAGAAC
H12	ACTTAT

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In This Book

This guide contains information to run the HaloPlex Target Enrichment System protocol.

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